Differential Assessment of Vascular Survival Ability and Tumor Angiogenic Activity in Colorectal Cancer

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ABSTRACT

Background: **The process of new vessel formation during neoplastic transformation and growth (neoangiogenesis) comprises proliferation, sprouting, and migration of endothelial cells within normal tissues adjacent to the tumor. These new vessels are directed toward the tumor invading edge and provide the bed for the subsequent growth of new tumor layers. We previously showed various degrees of decreasing vascular density in tumor layers once these lose contact with the normal tissue. This suggests that, apart from angiogenic factors, vascular survival factors contribute equally to the structure of the tumoral vasculature. This "vascular survival" potential can be assessed by comparatively examining the vascular density in peripheral and inner tumor areas.**

Experimental Design: **Using immunohistochemistry with the anti-CD31 monoclonal antibody, we assessed the tumor angiogenic activity (TAA) and vascular survival ability (VSA) in a sample of 242 patients with Dukes' stage A (90 patients), B (73 patients), and C (79 patients) colorectal cancer treated with surgery alone.**

Results: **Overall, High TAA and VSA were significantly related with poor prognosis ($P = 0.03$; hazard ratio, 1.9 and $P = 0.001$; hazard ratio, 2.7, respectively). In multivariate analysis, VSA was revealed as the most potent and independent prognostic factor ($P = 0.0001$; *t* ratio, 4.5), followed by vascular invasion ($P = 0.0001$; *t* ratio, 4.4) and stage ($P = 0.01$; *t* ratio, 2.5). Tumors with high TAA and high VSA had a significantly higher risk to develop liver metastasis ($P = 0.0003$).**

Conclusions: **Assessment of VSA in addition to TAA provides additional important prognostic information in patients with colorectal cancer and can be a useful tool in the recruitment of patients who would benefit from angiostatic *versus* angiotoxic therapies.**

INTRODUCTION

Angiogenesis is an important step in the process of tumor growth and invasion, as has been confirmed in studies dealing with the adenoma-carcinoma sequence in colorectal cancer (1) and in studies focusing on the transition from preinvasive mucosal to invasive to submucosa lesions (2). However, additional factors relevant to the proliferation/apoptosis balance, *e.g.*, cell-cell and cell-matrix adhesion, cancer cell motility and migration, and host immune response against the tumor, are of equal importance in defining tumor growth and its invasive and metastatic behavior. Recent studies also suggest that TAA[2] is not the only process related to the tumor vasculature that may influence prognosis. The degree of vascular maturation varies among tumors, and such a parameter is independent of MVD (3, 4). Furthermore, the ability to maintain the newly formed vasculature varies among tumors, so that tumors with highly angiogenic edges may have a very poor degree of vascularization in inner tumor areas. This was shown in our previous studies in non-small cell lung and in breast cancer (5, 6).

The intensity of this process, which we call VSA, can be assessed in tissue slides immunostained with a panendothelial cell marker in addition to the standard MVD method (5). VSA assessment is based on the concept that an immunostained section of a resected tumor does not give just a static image of the tumor at the time point of the resection, but rather reflects aspects of the history of the tumor from its growth until the time of surgery. During the growth process, the invading front of the tumor is gradually becoming an inner tumor area as newly invading cancer foci in the adjacent normal tissue are organized into an outer tumor layer. Because angiogenesis of successive tumor invading fronts should not vary during the course of tumoral growth (unless new clones appear), comparative examination of the vascular density in invading tumor layers *versus* inner layers gives an estimate not only of the angiogenic activity, but also of the survival ability of the tumoral vasculature, once the internalizing peripheral tumor layer loses contact with the adjacent normal tissues.

In the present study we provide strong evidence that TAA and VSA are independent processes in colorectal cancer and that high VSA is one of the most important stage-independent prognostic factors.

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[2] The abbreviations used are: TAA, tumor angiogenic activity; MVD, microvessel density; VSA, vascular survival ability; edvin, edge *versus* inner; HR, hazard ratio; VEGF, vascular endothelial growth factor.

Table 1 Patients and tumor characteristics

No. of patients	242
Age, median (range), yrs	67 (0–88)
Sex (M/F)	135/107
Location	
Rectum	147
Colon	95
Stage	
A	90
B	73
C	79
Histological grade	
1	88
2, 3	154
Vascular invasion	
No	197
Yes	45

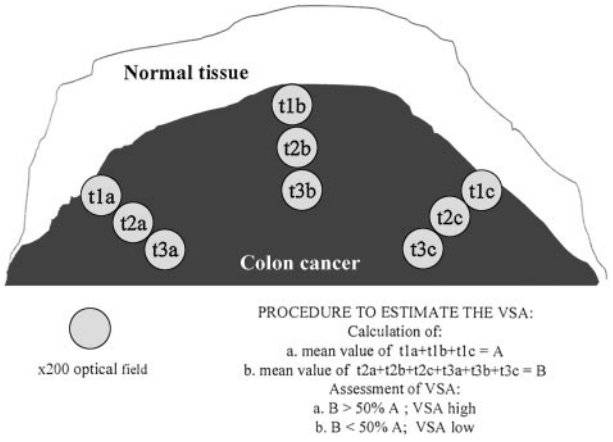

Fig. 1 Schematic representation of the procedure used to assess VSA.

MATERIALS AND METHODS

Paraffin-embedded tissue material from 242 patients with colorectal cancer treated with surgery alone was retrieved from the Pathology Department of Democritus University of Thrace (Alexandroupolis, Greece). Hematoxylin sections were used to choose tissue sections, where both tumor and normal colon was available, so that invading and inner tumor areas could be studied immunohistochemically.

Details on the patients and disease are reported in Table 1. Patients were treated with curative resection and anastomosis or permanent colostomy according to the feasibility of surgical maintenance of a functional anal sphincter. Patients with positive surgical margins or tumor close to the surgical margins (distance of tumor edges from the resection margins <3 cm) were excluded. Similarly, patients who received adjuvant chemotherapy or postoperative radiotherapy were excluded to avoid biases relevant to tumor chemo- or radiosensitivity. Patients who died within 30 days after surgery were also excluded to avoid bias from perioperative death. The follow-up of patients ranged from 1.4 to 99 months (median, 31 months).

Immunohistochemical Staining. The JC70 monoclonal antibody (Dako, Copenhagen, Denmark), which recognizes CD31 (platelet/endothelial cell adhesion molecule-1; Ref. 7) was used for microvessel staining on 3-μm paraffin-embedded sections according to the alkaline phosphatase-antialkaline phosphatase procedure. Sections were dewaxed, rehydrated, and predigested with protease type XXIV for 20 min at 37°C. JC70 as undiluted supernatant was applied at room temperature for 30 min and washed in Tris-buffered saline. Rabbit antimouse antibody diluted 1:50 was applied for 30 min, followed by application of alkaline phosphatase-antialkaline phosphatase complex (1:1, v/v) for 30 min. After sections were washed in Tris-buffered saline, the last two steps were repeated for 10 min each. The color was developed by a 20-min incubation with New Fuchsin solution (Dako). As a positive control, we used tissue sections from third-trimester chorionic villi, whereas normal mouse IgG was substituted for primary antibody at the same concentration as negative control.

Assessment of TAA. The immunostained tissue sections were assessed simultaneously by two pathologists (A. G. and E. S.) over the conference microscope. Each pathologist gave each section a score, and discrepancies were discussed and resolved. Both pathologists were blinded to the clinical outcome of the patients.

Table 2 Comparative analysis of the MVD in the tumor periphery and inner areas

Distribution of tumors according to TAA within different Dukes' stages.

Dukes' stage	MVD[a]			TAA[b] (no. of patients)	
	t1	t2	t3	Low	High
A	46 ± 12	29 ± 15	24 ± 14	48	42
B	46 ± 20	28 ± 17	22 ± 16	39	34
C	46 ± 21	28 ± 16	22 ± 17	40	39

[a] *P*: stage A, B, and C, t1 *vs.* t2, $P < 0.0001$; stage A, t2 *vs.* t3, $P = 0.02$; stage B, t2 *vs.* t3, $P = 0.05$; stage C, t2 *vs.* t3, $P = 0.04$.
[b] A *vs.* B *vs.* C, *P*, not significant.

Microvessel counting was used for angiogenesis assessment. Sections from primary tumors were scanned at low power (magnification, ×40 and ×100). Areas with the highest vascularization within the tumor invading front (adjacent to the normal colon) were chosen, and microvessel were counted in three chosen ×200 fields with the highest density. Microvessels adjacent to necrotic areas were excluded from the appraisal. The final MVD was the mean of the vessel counts obtained in these fields. The median MVD recorded in the tumors assessed was used as the cutoff point to define two groups of tumors with high and low TAA.

Assessment of VSA. Three areas of tumor adjacent to normal colon bearing the highest vascularization were identified per case. Microvessels were counted in three consecutive ×200 fields, starting from the tumor tissue adjacent to the normal colon (t1 field; tumor periphery), and moving the optical field twice toward the tumor center (t2 and t3 fields; called for convenience intermediate and inner tumor areas, respectively). These three fields (t1, t2, and t3) were estimated to cover a distance of 6 mm (~2 mm each) from the

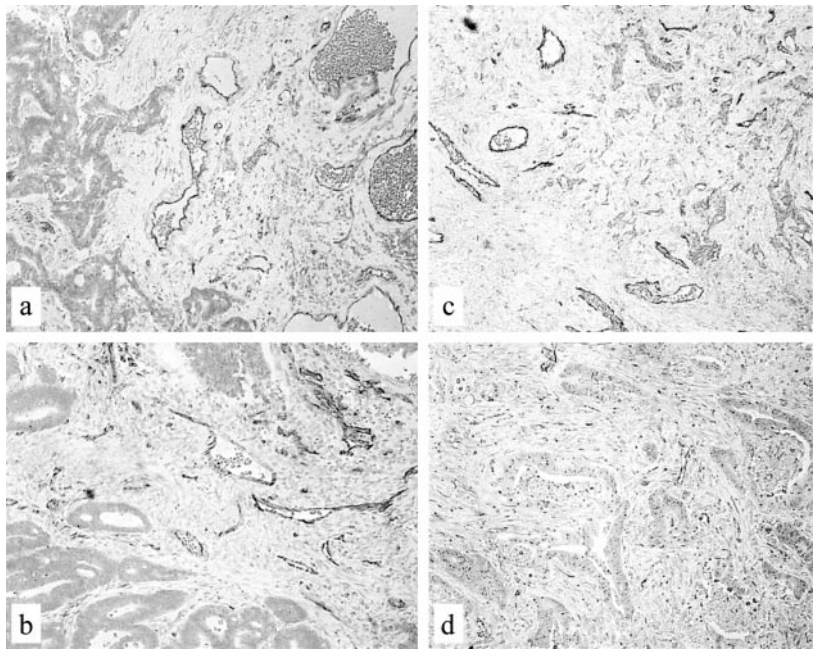

Fig. 2 *c* and *d*, a case of colon adenocarcinoma classified as edvin 3. Note the high vascular density in the invading front (t1 area; *c*), whereas a dramatic reduction occurs 4 mm away (t3 area; *d*). *a* and *b*, a case of colon carcinoma classified as edvin 4. Note the high vascular density in the invading tumor edge (t1 area; *a*), which is maintained in inner tumor layers (t3 area; *b*).

Table 3 Correlation between TAA and VSA

	VSA		
TAA	Low	High	*P*
Stage A			
Low	29	19	0.02
High	15	27	
Stage B			
Low	21	18	0.81
High	17	17	
Stage C			
Low	24	16	0.65
High	21	18	

Table 4 Correlation of edvin score with histological grade, vascular invasion, and with metastatis of the liver

	Edvin score				
	1	2	3	4	*P*
Histological grade					
1	32	21	11	24	3 *vs.* 1, $P = 0.008$
2, 3	42	32	42	38	3 *vs.* 2, $P = 0.03$
					3 *vs.* 4, $P = 0.03$
Vascular invasion					
No	64	43	39	51	1 *vs.* 3, $P = 0.06$
Yes	10	10	14	11	
Liver metastasis					
No	71	45	46	46	1 *vs.* 2, $P = 0.02$
Yes	3	8	7	16	1 *vs.* 3, $P = 0.05$
					1 *vs.* 4, $P = 0.0003$
					4 *vs.* 2, 3, $P > 0.09$

periphery to the center. The mean MVD in the peripheral, intermediate, and inner tumor areas was the mean value obtained from the three t1, three t2, and three t3 areas assessed, respectively (MVD_{t1}, MVD_{t2}, and MVD_{t3}, respectively). Fig. 1 shows a schematic of the procedure followed for the assessment of vascular density. Cases were divided into two groups according to their ability to maintain the MVD in inner tumor areas. Cases with a $MVD_{t1} < MVD_{t2+t3}$ (mean MVD in "t2 + t3" areas >50% of the mean MVD in the t1 areas) were considered to have a high VSA.

Statistical Analysis. Statistical analysis was performed and graphs were constructed using the GraphPad Prism 2.01 package. Survival curves were plotted using the method of Kaplan and Meier, and the log-rank test was used to determine statistical differences between life tables. A Fisher's exact test was used for testing relationships between categorical tumor variables. A Cox proportional hazard model was used to assess the effects of patient and tumor variables on overall survival. $P < 0.05$ was considered significant.

RESULTS

The mean MVDs per ×200 optical field in the invading (t1), intermediate (t2), and inner tumor (t3) areas according to stage are shown in Table 2. The MVD decreased significantly from the t1 to the t2 regions ($P < 0.0001$) and from the t2 to the t3 regions ($P < 0.05$) in all three Dukes' stages. No difference in MVD among Dukes' stages was noted in any of the three t areas.

Using the median MVD in t areas as a cutoff point, we grouped patients into two categories of low and high TAA, as shown in Table 2.

Analysis of VSA and "Edvin" Groups. Tumors with a mean MVD in the t2 + t3 areas that exceeded 50% of the MVD in the t1 area were considered to have a good ability to maintain a relatively high degree of vascularization in inner tumor areas. Fig. 2 shows the comparative immunohistochemical staining of two cases with high *versus* low VSA.

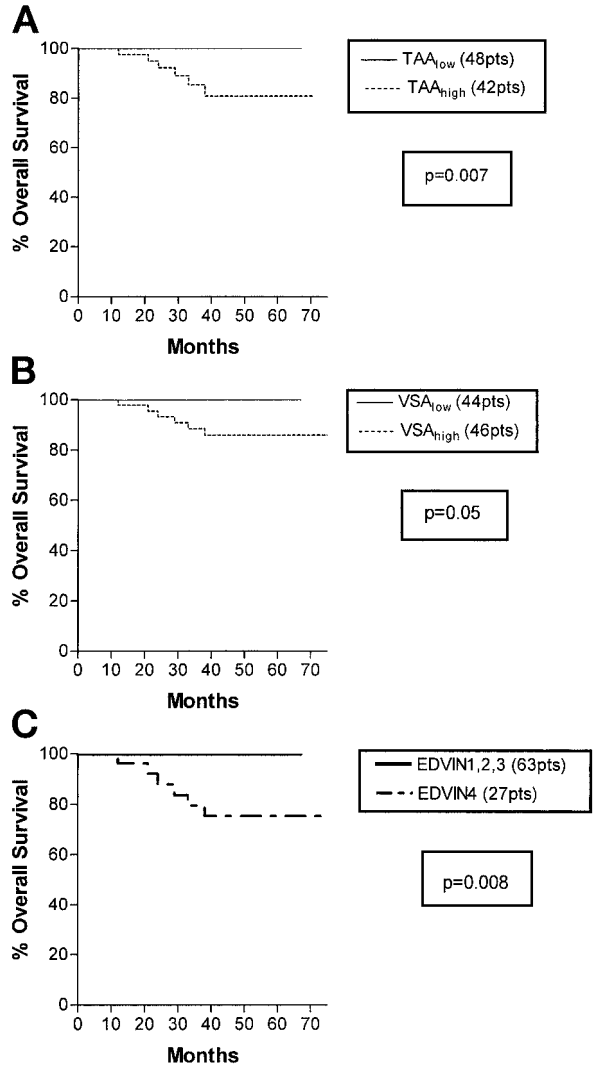

Fig. 3 Kaplan-Meier curves for overall survival in Dukes' stage A patients, stratified for TAA (*A*), VSA (*B*), and edvin groups (*C*). *pts*, patients.

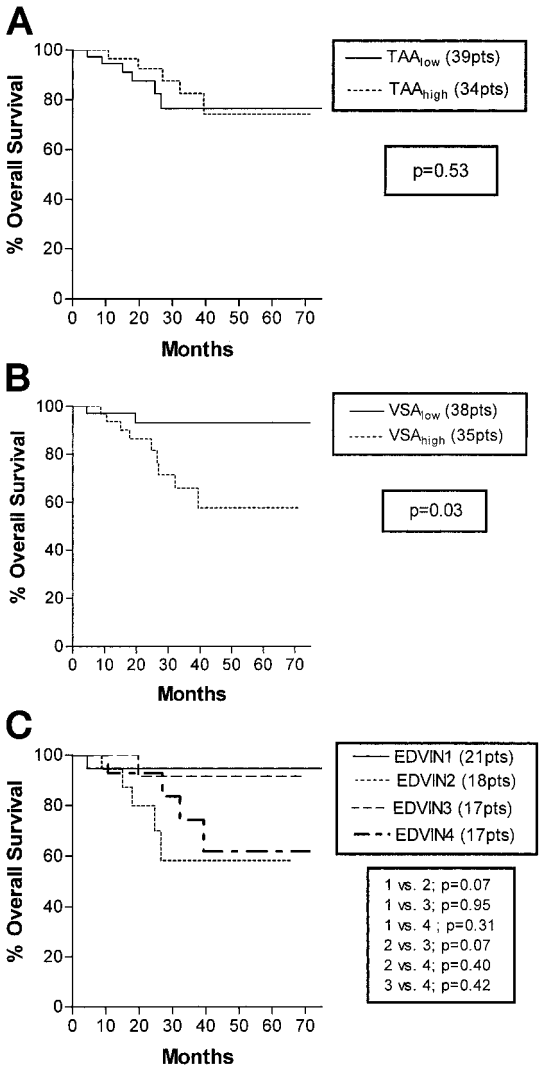

Fig. 4 Kaplan-Meier curves for overall survival in Dukes' stage B patients, stratified for TAA (*A*), VSA (*B*), and edvin groups (*C*). *pts*, patients.

Shown in Table 3 is the distribution of cases according to the TAA and the VSA. Using these two variables, we were able to distinguish four different edvin groups (5): (*a*) tumors with high TAA and high VSA (edvin 4); (*b*) tumors with high TAA and low VSA (edvin 3); (*c*) tumors with low TAA and high VSA (edvin 2); and (*d*) tumors with low TAA and low VSA (edvin 1). High TAA was significantly linked with high VSA only in stage A tumors, whereas no such association was noted in stages B and C.

Correlation of Edvin Score with Histological Variables. Table 4 shows the analysis of the edvin score according to the histological grade, vascular invasion, and the development of hepatic metastasis. Edvin 3 cases were significantly linked to high histological grade. Although edvin 1 cases less frequently showed invasion of cancer cells into the vascular spaces, the difference was not significant. Edvin 1 cases had a significantly lower ability to metastasize to the liver compared with the other groups. The risk of liver metastasis was 2- and 5-fold increased in edvin 4 cases compared with edvin 2-3 and edvin 1 cases, respectively.

Further analysis of the size of the primary tumor did not reveal any association with the edvin score. Although stage-C/edvin-1 cases had lower mean numbers of involved nodes compared with other edvin groups, the difference did not reach significance ($P > 0.15$).

Univariate Analysis of Survival. In univariate analysis Dukes' stage ($P < 0.009$; HR, 3.3–9.6), histology grade ($P = 0.0004$; HR, 3.6), and vascular invasion ($P < 0.0001$; HR, 6.8) were significant prognostic variables, whereas tumor location (rectum *versus* colon) was not ($P = 0.38$; HR, 1.2). High TAA and VSA were significantly related with poor prognosis ($P = 0.03$; HR, 1.9 and $P = 0.001$; HR, 2.7, respectively).

We further analyzed the prognostic role of TAA, VSA, and edvin score within the three Dukes' stages (Figs. 3 –5). TAA

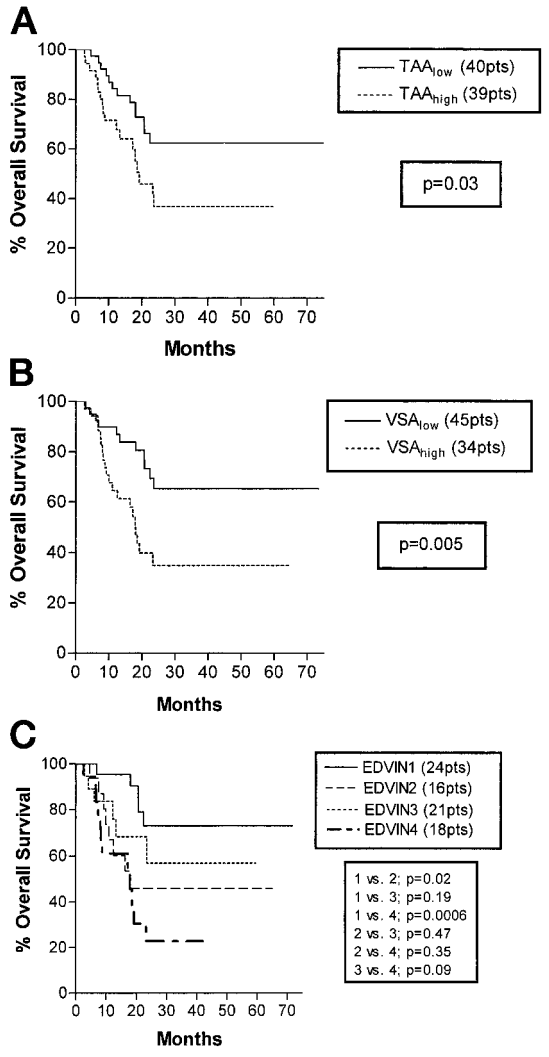

Fig. 5 Kaplan-Meier curves for overall survival in Dukes' stage C patients, stratified for TAA (*A*), VSA (*B*), and edvin groups (*C*). *pts*, patients.

and VSA were significantly related to poor survival in patients with stage A and C. In stage B, VSA (but not TAA) was related with poor outcome.

In stage A, the patients with edvin 4 were the only group related to poor survival. In stage B, edvin 2 and 4 had a marginally significant association with poor outcome. In stage C, edvin 2 and 4 exhibited a significantly worse outcome compared with other edvin scores.

Multivariate Analysis. The multivariate analysis of death events for all cases and within the different Dukes' stages are shown in Table 5. Overall, VSA was the most significant independent prognostic variable, followed by vascular invasion and Dukes' stage.

DISCUSSION

TAA has been recognized as a major factor defining local and distant recurrence of the disease after surgery. The adverse prognostic role of angiogenesis has been also confirmed in several clinicopathological studies in colorectal cancer (8–19). Four studies also report that high MVD is associated with distant metastases in colorectal cancer (11, 12, 16, 20). Nevertheless, reports that failed to confirm an association of high MVD with poor prognosis in colorectal cancer have also been published (21–23), including an early large study by Bossi *et al.* (24). In a study by Pavlopoulos *et al.* (25), the MVD was not related to prognosis, whereas a significant association of the "total vascular area" as assessed by computer image analysis was noted. Still, a recent report by Abdalla *et al.* (26) suggests that high MVD is related to a better clinical outcome.

In the present study we assessed MVD in three hot-spots that were persistently found in the tumor invading front. This represented the TAA. Indeed, endothelial cell proliferation occurs predominantly in the tumor periphery, as shown by Fox *et al.* (27). Once the new vessels are formed in the tumor edge and gradually incorporated by the growing tumor, their ability to survive becomes an important factor that will define the vascular density within the tumor body. Several factors involved in endothelial cell apoptosis inhibition, *i.e.*, VEGF, bcl2, and survivin (28–30), could be part of such a process. Comparative analysis of the MVD in the invading tumor edge *versus* inner areas allowed the appraisal of the VSA, as described previously (5).

Analysis within stages showed that the TAA was not different among stage A, B, and C, suggesting that the angiogenic tumor activity reaches a maximum in early stages of the development of the disease. This is in accordance with a previous study by Pavlopoulos *et al.* (25), in which the MVD was higher in early phases of development of colorectal cancer, whereas this decreased with progressing Dukes' stage. We also noted that the mean MVD drops rapidly within a distance of 2–4 mm from the invading tumor edge and decreases less sharply thereafter in deeper tumor layers. The TAA varied widely among tumors: the MVD ranged from 10 to 117 microvessels per ×200 optical field. The VSA also varied among cases: the MVD in the t1 areas was 1–7.3 times and 1–14 times higher than in the t2 and t3 areas, respectively.

Overall, no association between TAA and VSA was noted, suggesting that the biological pathways controlling angiogenesis are not identical to the ones controlling vascular survival. In a previous study in non-small cell lung cancer, we found that the maturation status of the tumoral vasculature (presence of lamina lucida) depended on the specific angiogenic profile of tumors, in that thymidine phosphorylase expression in absence of VEGF expression was linked to poor maturation status (3). A relevant finding has been reported in another of our studies, in which the ability of tumors to maintain high MVD in inner tumor layers was strongly related to VEGF expression but not to thymidine phosphorylase (5).

Using the TAA and the VSA, we divided tumors in 4 edvin groups. Edvin 3 tumors (with high TAA and low VSA) were linked to poor differentiation and high incidence of vascular invasion. Reduced vascular maturation, compatible with impaired VSA and enhanced vulnerability of vessels to cancer cell invasion, may account for this finding. On the other hand, edvin 4 tumors (high TAA and high VSA) had the highest incidence of distant metastasis to the liver. These findings may show that

Table 5 Multivariate analysis of death events

	Stage							
	All		A		B		C	
Variable	*P*	*t* ratio	*P*	*t* ratio	*P*	*t* ratio	*P*	*t* ratio
Stage (C *vs.* B *vs.* A)	0.01	2.5						
Grade (2, 3 *vs.* 1)	0.08	1.7	0.001	3.2	0.90	0.11	0.37	0.80
Location (rectum *vs.* colon)	0.13	1.4	0.20	1.2	0.97	0.03	0.71	0.36
Vascular invasion (yes *vs.* no)	0.0001	4.4			0.30	1.04	0.002	3.10
TAA (high *vs.* low)	0.12	1.5	0.10	1.6	0.83	0.20	0.25	1.14
VSA (high *vs.* low)	0.0001	4.5	0.0001	3.5	0.01	2.50	0.007	2.71

although angiogenesis is required for vascular invasion and release of cancer cells into the blood stream, cancer cells able to produce tumors with a high VSA have a higher chance to survive, establish, and produce viable colonies in distant organs. This is further supported by the finding that although TAA and VSA were important prognostic variables in colorectal cancer, edvin 4 cases (high TAA and high VSA) had a particularly poor survival. In multivariate analysis, VSA was the most important independent prognostic variable even within each of Dukes' stages separately. The value of TAA was strongly diminished in multivariate models that included VSA.

We conclude that assessment of VSA provides important prognostic information in colorectal cancer treated with surgery, which is independent of stage and TAA. The differential assessment of TAA and VSA may further be of value in identifying patients who would benefit from angiostatic or angiotoxic therapeutic approaches.

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